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Unmasking silent neurotoxicity following developmental exposure to environmental toxicants

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ABSTRACT

Silent neurotoxicity, a term introduced approximately 25 years ago, is defined as a persistent change to the nervous system that does not manifest as overt evidence of toxicity (i.e. it remains clinically unapparent) unless unmasked by experimental or natural processes. Silent neurotoxicants can be challenging for risk assessors, as the multifactorial experiments needed to reveal their effects are seldom conducted, and they are not addressed by current study design guidelines. This topic was the focus of a symposium addressing the interpretation and use of silent neurotoxicity data in human health risk assessments of environmental toxicants at the annual meeting of the Developmental Neurotoxicology Society (previously the Neurobehavioral Teratology Society) on June 30th, 2014. Several factors important to the design and interpretation of studies assessing the potential for silent neurotoxicity were discussed by the panelists and audience members. Silent neurotoxicity was demonstrated to be highly specific to the characteristics of the animals being examined, the unmasking agent tested, and the behavioral endpoint(s) evaluated. Overall, the experimental examples presented highlighted a need to consider common adverse outcomes and common biological targets for chemical and non-chemical stressors, particularly when the exposure and stressors are known to co-occur. Risk assessors could improve the evaluation of silent neurotoxicants in assessments through specific steps from researchers, including experiments to reveal the molecular targets and mechanisms that may result in specific types of silent neurotoxicity, and experiments with complex challenges reminiscent of the human situation.

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1. Introduction

"Silent neurotoxicity", also called silent toxicity or silent damage to the nervous system, represents a persistent biochemical change or morphological injury to the nervous system that does not induce overt evidence of toxicity (i.e. remains clinically unapparent) unless unmasked by experimental or natural processes (Reuhl, 1991; Grandjean, 2008; Giordano and Costa, 2012). This concept has been described in association with numerous neurotoxic chemicals and neurodegenerative diseases since its first use approximately 25 years ago (Needleman, 1993; Weiss, 1996; Thiruchelvam et al., 2002; Weiss et al., 2002; Costa et al., 2004; Cory-Slechta et al., 2005; Barlow et al.,

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2007; Fan et al., 2011). While silent neurotoxicity might be relevant to a broad range of toxicity mechanisms, and therefore be of concern for many environmental chemicals, it remains poorly understood.

Silent neurotoxicity overlaps with other commonly used terms, including, perhaps most closely, the "2-hit" or "multiple hit" models and hypotheses (including the "neurodevelopmental hypothesis"). Each of these terms describe the occurrence of initial insults that can alter cellular function and prime a system to either make it vulnerable to a subsequent insult(s) or progressively lead to loss of normal function with additional insult(s), with the effects of one insult in isolation being insufficient to induce disease. These theories are based on studies of carcinogenesis, but have been more recently applied to nervous system effects (Nordling, 1953; Armitage and Doll, 1957; Weinberger, 1987; Bayer et al., 1999). Silent neurotoxicity is also related to the phenomena of chemical and non-chemical "interactions" (National Research Council, 2009) and "latent" or "delayed" neurotoxicity (Aldridge et al., 1969). In general, although there may be slight differences in the application of these closely related terms (e.g., emphasis on a period of latency between exposure and the ability to reveal effects; emphasis on a developmental insult; limited to specific types of "unmasking"; etc.), all of these inter-related concepts are attempting to examine and describe neurotoxicity in terms of cumulative effects that may be more relevant to human exposure situations than traditional toxicity testing for effects of a single chemical measured immediately after exposure.

For silent neurotoxicity, in the context of environmental health, a factor that "unmasks" toxicity could be any stimulus that challenges or otherwise adjusts the threshold of a cellular system that is the target of a particular environmental agent. Some examples of "unmasking" agents may include stress, disease, infection, or multiple chemical exposures, while related natural processes could include aging or loss of tolerance. Although the topic of silent neurotoxicity does not represent a new concept (Reuhl, 1991), it is one that persists in the neurotoxicology literature (Giordano and Costa, 2012). Despite the attention given to this topic, our current understanding of the different causes for silent neurotoxicity remains incomplete. While recent research continues to unravel the molecular targets that may be involved for certain types of insults, which is expected to improve our understanding of the molecular mechanisms of developmental neurotoxicity and help to move the field forward (e.g., via construction and sharing of common adverse outcome pathways (Ankley et al., 2010)), identifying potential silent neurotoxicants remains difficult for risk assessors.

This topic was the focus of a symposium held on June 30th, 2014 at the 38th annual meeting of the Developmental Neurotoxicology Society (previously the Neurobehavioral Teratology Society) held in Bellevue, WA from June 28th–July 2nd. The symposium was organized into a series of presentations followed by a facilitated panel discussion with the audience, which was comprised of academicians, federal and state government scientists, industry scientists, and non-government organization representatives. The intent of the discussion was to provide information useful to risk assessors, who often find these data (particularly data related to behavioral changes) difficult to incorporate into their neurotoxicity hazard descriptions. It is clear that the appropriate interpretation of experiments capable of revealing silent neurotoxicity would also be of critical use to decision-makers considering cumulative risk assessment (i.e. the combined risk from multiple agents or stressors, including chemical mixtures as well as non-chemical stressors) and environmental justice scenarios (e.g., improving the decision maker's assessment of effects from disproportionate exposure in low income communities by effectively identifying the most vulnerable subpopulations), two key areas of current interest in the risk assessment field.

From a risk assessment vantage-point, the potential for silent neurotoxicity is concerning for a number of reasons. First, because these types of experiments can be difficult to perform, they are seldom found in the published literature and, when available, they are often focused on narrowly defined, specific research questions that can be difficult to extrapolate to broader exposure scenarios or populations being investigated by the risk assessors. Additionally, experimental animal studies conducted according to guideline protocols (e.g., a developmental toxicity study), which may be viewed as the most desirable studies available for use in risk assessments due to factors such as their use of standardized endpoints and large group sizes, are not designed to detect these types of effects. Taken together, the fact that the overwhelming majority of studies used in risk assessments do not consider multiple insults, or "challenges", in a single model has the potential to result in an underrepresentation of the true potential for developmental neurotoxicity induced by the substance(s) under evaluation. For environmental agents that only exhibit toxicity in the presence of another chemical or non-chemical stressor, if experimental unmasking challenges were not examined, then a neurotoxicity hazard could be considered unlikely based on evidence collected under basal conditions. Since every human exposure scenario involves both chemical and non-chemical stressors, with the right combination the chemical might exhibit effects that wouldn't be identified by experimental studies testing for effects in isolation. Similarly, effects that appear to be "reversible" (i.e. phenotypes that appear to be associated with the presence of the causal agent in the body) may not be used for derivation of toxicity values, even though these agents may have additional latent effects observed only with unmasking. In addition to laboratory-based toxicology experiments, silent neurotoxicity also poses a difficulty for epidemiologic studies. For instance, it might be problematic to link an observed apical effect back to a particular chemical exposure if the investigator does not specifically examine effect modification by the unmasking stimuli revealing the response (e.g., age; other chemical exposures). Finally, a lack of data examining silent neurotoxicity increases the possibility of assessors providing an incomplete description of potentially sensitive subpopulations and lifestages, such as children who experienced prenatal maternal stress (e.g., acute or chronic distress during pregnancy that may result from emotional or physical trauma to the mother) or individuals exposed to a complex mixture containing both the contaminant in question and other agents capable of unmasking neurotoxicity. Overall, this continues to represent a critical and controversial topic for neurotoxicity risk assessment.

2. Summary and critical messages from the symposium presentations

Dr. Aschner introduced the topic by presenting human data from methylmercury (MeHg) poisonings in Minamata, Japan (contaminated fish) and Iraq (contaminated grain). He reflected that exposed victims consistently exhibited a long latent period of weeks to months (or longer) after exposure ended before the onset of behavioral symptoms (Grandjean et al., 1998; Myers et al., 2000; Castoldi et al., 2003). Initially, these latent effects were hypothesized to be due to the slow accumulation of a toxic metabolite, iHg (inorganic mercury), which would vary across individuals depending on their metabolism, and which would be expected to correlate inversely with latency. One would expect the buildup of iHg to be faster at higher levels of MeHg exposure, resulting in a shorter latency period. However, this is not substantiated by the data, with patients experiencing higher MeHg exposure levels failing to show shorter latency periods (Weiss et al., 2002). Interestingly, in at least some cases, the onset of different behavioral phenotypes was shown to occur with different latencies (e.g., visual constriction could be manifest several months after ataxia). While this may illustrate progressive injury to a single cellular system, which seems unlikely for the example above at least, it is probably more likely that this disparity reflects differences in the time-dependence for clinically manifest changes due to effects at different molecular targets, including the dopamine and GABA neurotransmitter systems (Newland et al., 2008). It is also possible that the rate of conversion of MeHg to iHg is rate-limited and therefore iHg is constantly generated at a rate independent of the level of MeHg; however, the literature does not support the underlying

assumption that iHg is the proximate toxic agent in MeHg poisoning (Magos et al., 1985).

This latency requirement for observations of behavioral neurotoxicity illustrated the need for risk assessors to keep in mind the complexity of human disease, a topic picked up by Dr. Cory-Slechta. Dr. Cory-Slechta emphasized that human disease is a consequence of multiple, co-occurring risk factors across time. She urged researchers to consider the use of behavioral measures common to human scenarios, as complex and multifactorial tests are likely to best represent the human condition. A research-specific example she provided loosely equated fixed interval performance testing to the typical human scenario of studying for an exam, and cramming as the exam approaches. Dr. Cory-Slechta also emphasized that it is essential to consider common adverse outcomes and common biological targets for chemical and non-chemical stressors, particularly when these insults are known to co-occur. For instance, both lead toxicity and prenatal stress appear to target the hypothalamic-pituitary-adrenal (HPA) axis and the mesocorticolimbic system, which partly mediate cognition and attention (Piazza et al., 1996; Cory-Slechta et al., 1998). This type of consideration led to a series of studies by Dr. Cory-Slechta on the effects of lead and prenatal stress on these systems and behaviors. This idea of conserved molecular or cellular targets for the various chemical challenges and stressors encountered by an organism at different stages of development was also highlighted during presentations by Drs. Aschner and Caudle, and Dr. Bilbo similarly described the concept of "2-hits" to the same target(s). Drs. Aschner and Caudle described the common idea of stress or insults during development causing a change in the vulnerability or trajectory to disease in the adult organism. They discussed how early developmental insults could change the threshold for the onset of symptoms (i.e. Parkinson's disease (PD)-like symptoms) by causing a subclinical change in the neural cells (i.e. dopaminergic neurons) or neurochemistry involved. They noted that subclinical developmental modifications might accelerate the trajectory of normal, later-life disease states, or increase the vulnerability of certain systems to future unmasking insults which are then able to overcome the plasticity-related responses of the brain (Richardson et al., 2006; Richardson et al., 2008; Racette et al., 2012). Dr. Cory-Slechta suggested a similar idea in that early adverse behavioral conditions may be associated with a less resilient, more vulnerable phenotype leading to a sensitive system. In the context of environmental justice, these lines of research might help to identify early changes in subpopulations of disadvantaged communities that may be at increased risk of neurotoxicity with chemical exposure later in life.

The variable range of potential unmasking agents that might be relevant to assessing silent neurotoxicity was apparent when looking across the presentations. Experiments by Drs. Cory-Slechta and Bilbo both demonstrated that behavioral changes related to anxiety and cognition were sometimes only affected by the combination of exposure to an environmental toxicant (i.e. lead, MeHg or air pollution) and experimentally induced early life stress (i.e. prenatal nest restriction or immobilization) in rodents (Cory-Slechta et al., 2008; Bolton et al., 2013; Weston et al., 2014a). Dr. Aschner demonstrated that differences in genetic susceptibility may influence the likelihood of observing latent neurotoxicity in a *Caenorhabditis elegans* (*C. elegans*) model of PD (Bornhorst et al., 2014; Chen et al., 2015), while Dr. Caudle showed that exposure to one potential dopaminergic toxicant throughout gestation and lactation- the chlorinated organophosphate flame retardant, Tris (1,3-dichloroisopropyl)phosphate (TDCPP), can modify the vulnerability of the adult dopamine system to toxicity caused by exposure to a second chemical toxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), in adulthood (unpublished results). In Dr. Caudle's "2-hit" model, the animals exhibited an increased vulnerability to MPTP, with developmental TDCPP exposure enhancing the decreased locomotion and striatal markers of dopaminergic neuron function caused by MPTP, which is consistent with published results from his lab using endosulfan (Wilson et al., 2014). Dr. Bilbo also illustrated how postnatal changes in diet (i.e. a high-fat diet) can unmask neurotoxic changes caused by gestational exposure to air pollution (Bolton et al., 2014). An important point made throughout these presentations is that the type of unmasking agent matters. The data indicate that different types of unmasking agents (e.g., social; chemical; physical) may be required to elicit neurotoxicity in a chemical- and target system-specific manner. Therefore, not observing silent neurotoxicity after chemical exposure using a single stressor and a single behavioral measure does not mean that the same chemical exposure would not result in toxicity if another behavior or stressor was tested. Further, it should be recognized that even when different unmasking agents appear to act on the same system or molecular target, it is possible that they will unmask silent neurotoxicity in disparate ways.

Another interesting phenomenon was raised in the presentations by Drs. Cory-Slechta and Bilbo. These researchers observed a clear difference in sensitivity to silent neurotoxicity across sexes, which was dependent on the specific combination of chemical exposure, unmasking agent, and behavioral test(s) employed. Using prenatal exposure of mice to diesel exhaust particles (DEPs), Dr. Bilbo showed that DEP alone doesn't impact adult anxiety tested in a zero maze, but DEP with nest restriction stress to the dams increased measures of anxiety in fear conditioning and zero maze assays of the offspring (Bolton et al., 2013). In all of these experiments, the female pups were noticeably less vulnerable than the male pups in the behavioral assays. When Dr. Bilbo applied a second paradigm, namely prenatal DEP and postnatal administration of a high fat diet as an unmasking agent, once again the male pups, but not the female pups, were vulnerable, exhibiting increased anxiety at four weeks of age (Bolton et al., 2014). Interestingly, in Dr. Cory-Slechta's experiments employing developmental exposure to either lead or methylmercury, and a different paradigm of developmental stress as an unmasking agent (i.e. maternal stress induced by restraint), sex-dependent responses were also observed; however, in these experiments, effects on higher cognitive function were generally manifest in the female pups, not the male pups (Weston et al., 2014a; Weston et al., 2014b). It is unknown whether these differences in vulnerability across sexes are attributable to the different environmental chemical exposures, the different unmasking challenges, the different behavioral test paradigms, or some combination of these variations across experiments. Regardless, the data strongly suggest a need for examination of both sexes when evaluating the potential for silent neurotoxicity, similar to sentiments in related neuroscience research fields (e.g., (Beery and Zucker, 2011)).

Molecular changes which may be detectable prior to overt manifestations of neurotoxicity (e.g., during the latent period) is a critical area of ongoing and future research. Although these early events are often adaptive in nature, an increased understanding of the temporal progression of underlying neurobiological changes could eventually yield predictive insight. For the particular toxicants and endpoints discussed by the presenters, common molecular changes included alterations to the HPA axis, inflammation-related responses and oxidative stress. Dr. Aschner discussed early life MeHg exposure in a *C. elegans* model relevant to PD. The combination of exposure and knockout of *pdr-1*, a homologue to the human *parkin* gene, resulted in increased reactive oxygen species (ROS), which he hypothesized could be related to the later life neurotoxicity observable in this model (Chakraborty et al., 2015). Similarly, using in vivo and in vitro rodent models related to PD, Dr. Caudle observed suggestive associations between oxidative stress-related events and the development or progression of neurotoxicity (Caudle et al., 2007). Drs. Bilbo and Cory-Slechta both observed an association between altered HPA axis function and altered behavior. Dr. Bilbo observed that, in response to nest restriction, the male pups, but not the female pups, exhibited increased corticosterone (Bolton et al., 2013). Notably, the male pups were selectively vulnerable (i.e. only male pups exhibited impaired cognition) in these experiments. Dr. Cory-Slechta was also able to detect increased corticosterone in the blood, which was correlated with other molecular evidence of nervous

system changes, including decreases in the dopamine metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), in the prefrontal cortex (Cory-Slechta et al., 2010). Inflammation may also be complicit in these types of changes. The vulnerable male pups in Dr. Bilbo's experiments combining nest restriction and DEP exposure exhibited increased evidence of inflammation, such as increased expression of toll-like receptor 4 and caspase-1, whereas the female pups actually exhibited increased expression of anti-inflammatory interleukin 10 (Bolton et al., 2013). Additionally, the combination of exposure and dietary stress caused morphological and molecular markers of activation of microglia in the hypothalamus of the male, but not female, offspring. Dr. Bilbo went on to show that this increased mononuclear cell activation in the brain was, at least in part, due to macrophage infiltration into the brain, suggesting that there may be a linkage between changes in the adipose tissue and neuroinflammation in the brain (Bolton et al., 2014). Given these data and other similar findings emerging in the literature, the potential role for neuroimmune interactions and low level oxidative stress-induced changes in the development of silent neurotoxicity deserves additional study.

Several speakers briefly touched upon the issue of dose-response relationships for effects related to silent neurotoxicity. Dr. Cory-Slechta reminded the audience that not all stress is negative. She illustrated that the physiological response to some stressors often involves some adaptation or resilience at lower concentrations, which is overcome at higher concentrations. Dr. Aschner discussed a similar concept, noting that adaptive measures initiated in response to exposure during development typically engender short term benefits, such as making an individual better prepared to respond to an adverse environment. These presenters noted how non-monotonic responses are common, not only for measures of behavior, as one might expect, but also for the underlying neurochemical changes. Dr. Cory-Slechta illustrated this using exposure to MeHg and prenatal stress as an example, with offspring exhibiting non-monotonic changes in dopamine and serotonin metabolites, which differed by sex and brain region analyzed (Weston et al., 2014a). Interestingly, neurochemical alterations were observable in both sexes, even though only the females demonstrated behavioral changes, perhaps reflecting an increased resiliency of the associated system(s) in males. In addition to considering the potential for additivity of toxicants focused on the same biological system, Dr. Caudle emphasized that it is also important to recognize that these responses should not be expected to exhibit linear functions.

Across these presentations, measurements of changes in behavior that might be associated with silent neurotoxicity were shown to be dependent on a diverse set of variables. Previous work has demonstrated that stimuli during specific, sensitive periods of early life can have permanent consequences (e.g., Godfrey and Barker, 2001). Central nervous system (CNS) structures and functions undergo programming across various stages of embryonic, fetal, and early postnatal development (Selevan et al., 2000; Semple et al., 2013). This developmental programming determines the set points of physiological and metabolic responses in adult life. Depending on the timing, duration, and toxicant type, chemical exposure may lead to developmental adaptations (e.g., changes to CNS hormones or neurotransmitters; alterations in neuronal proliferation or migration) that readjust developmental set points. As many of these set points are governed by the anatomical location of the target cells, or in response to genes that are only expressed during a limited developmental window, this raises additional possibilities related to brain region-specific vulnerability and potential differences in genetic susceptibility for different silent neurotoxicants. Due to the adaptability of the developing CNS, alterations in specific neuronal nuclei may not result in an overt change in phenotype during development. Yet, these same changes may predispose the adult to aberrant physiological functions and increased risk of disease as the CNS is further challenged, either with normal aging or by other unmasking agents.

Fig. 1 attempts to illustrate some of the concepts that are likely to be important for neurotoxicity risk assessors to consider. In this simplified example, hypothetical changes in undefined functional markers

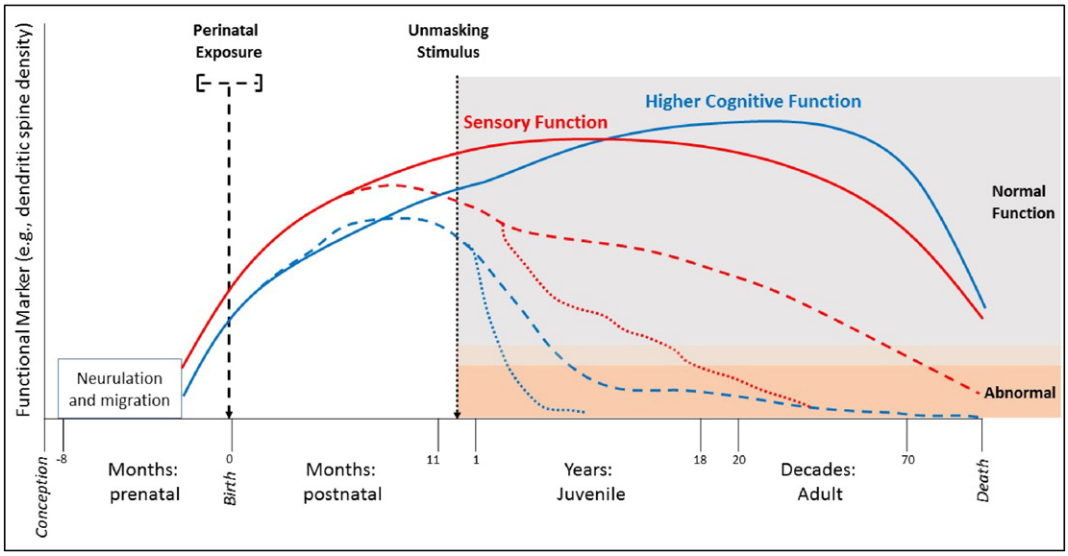

Fig. 1. Hypothetical effects of developmental exposure and unmasking stimulus on lifetime neurological function. This schematic illustrates the potential lifetime functional trajectory of two theoretical sets of neurons, namely those responsible for sensory function (solid red line) and those responsible for higher cognitive function (solid blue line). Over time, both sets of neurons undergo a prescribed, programmatic increase in functional marker(s) reflecting the development of those specific behaviors until some optimal level is achieved. These markers could theoretically include region-specific changes in neuron number, neurotransmitter levels, and/or synapse number or dendritic spine density (or other markers). A hypothetical range of functional marker levels necessary for "normal" behavior is illustrated (gray shading), with a theoretical threshold below which "abnormal" behaviors are observable (orange shading). Perinatal exposure to a toxicant for a brief time prenatally and postnatally (dashed black line) results in an eventual change in the trajectory of both sensory function (dashed red line) and higher cognitive function (dashed blue line). If this perinatal exposure is combined with exposure to an unmasking stimulus in late infancy, this trajectory is further modified (for sensory function: dotted red line; for higher cognitive function: dotted blue line). This conceptual illustration is adapted primarily from concepts presented in Thompson and Nelson (2001) (doi: 10.1037/0003-066X.56.1.5), with modifications based on reviews by Landrigan et al. (2005) and Catts et al. (2013).

(e.g., measures of synapse strength) for two different behavioral phenotypes are shown. For both behaviors, although the specific timing and magnitude of the trajectories differ, the functional markers are depicted as increasing up until at least early school age, with lesser increases through adolescence, after which the markers plateau and eventually decline at later ages. Although a wide range for "normal function" is illustrated, depending on the marker(s), optimal function could be achieved at the lower end of that range (e.g., for a marker such as synapse number, synaptic refinement and experience-dependent learning would be expected to result in a smaller number of stronger synaptic connections). Likewise, while the figure depicts a theoretical threshold in the functional markers below which clinically apparent abnormalities in behavior can be detected, excesses or overactivation of some nervous system components, and not just decrements, can also indicate toxicity. In Fig. 1, exposure to an undefined toxicant during the perinatal period has a more robust effect on the trajectory of one phenotype (i.e. higher cognitive function; trajectory after exposure shown as dashed blue lines) than the other (i.e. sensory function; trajectory after exposure shown as dashed red lines). In addition to the specific behavioral phenotype assessed, this figure illustrates that time after exposure, or aging, is important to consider as a second variable in this comparison since abnormalities in either function are not observed directly after exposure. As the exposed subjects begin to age, it appears likely that an experimenter will be able to detect abnormalities in higher cognitive function, whereas declines in sensory function induced by exposure are not robust and abnormalities are not apparent until old age, making detecting these effects more difficult. This difference in sensitivity is assumed to be dependent not only on the target system(s) of the toxicant, but also on the timing (and possibly duration) of exposure. In Fig. 1, the concept of experimental unmasking is illustrated by introduction of an undefined unmasking agent sometime during infancy, with unmasking in the absence of perinatal exposure not causing any changes in the trajectory of these behaviors (not illustrated in Fig. 1). Again, depending on the type and timing of this unmasking insult, effects on one function or with one type of chemical exposure may be different from other scenarios. In this illustration, both sensory function and higher cognitive function are sensitive to this particular unmasking, resulting in an earlier onset of observable abnormalities for both functions. Again, the timing at which the behaviors are measured after unmasking is critical. For example, following the combination of exposure and unmasking, measuring sensory function at 5 years of age would reveal no abnormalities, whereas assessing sensory function at 20 years of age would reveal this silent toxicity. This simplified figure fails to capture the potential impact of several other important variables, including profound differences in sensitivity due to interindividual variations (e.g., across sexes or genetic backgrounds), exposure level-dependent differences in how the functional trajectories are altered, or possible biphasic-type responses to unmasking (e.g., adaptive responses to low levels of stress which might actually attenuate the declining trajectory caused by perinatal exposure). In all, researchers and risk assessors could benefit from considering the potential influence of a complex and diverse range of biological variables when designing and interpreting experiments related to silent neurotoxicity.

3. Discussion and considerations for future efforts

This discussion is framed around a series of topics discussed with the audience during a panel session at the symposium. Some common themes were raised during the speaker presentations, including consistent findings of sex-related differences in the manifestation of latent behavioral responses and the potential involvement of inflammation-related and/or oxidative stress-related responses as mechanistic events leading to the development of these phenotypes. All of the panelists discussed silent neurotoxicity as clearly adverse and of significant concern for the purposes of human health risk assessment, and they noted that traditional methods generally do a poor job of revealing it. As a result, this might raise a public health concern that adverse neurodevelopmental effects could be going undetected for environmental agents assessed based on traditional toxicity studies. However, the relative difficulty of measuring neurotoxic effects that can remain clinically undetectable for months or even years after exposure highlights the need not only for increased testing for such effects, but also for approaches to prioritize testing of agents that might be viewed as more likely to cause this form of toxicity (e.g., based on observations from structurally related chemicals; for chemicals with substantial human exposures at developmental ages established as periods during which regions of the nervous system are sensitive to lasting changes in structure and/or function). Although silent neurotoxicity has consistently received attention in the past scientific literature (Reuhl, 1991; Bondy and Campbell, 2005; Grandjean, 2008; Giordano and Costa, 2012), it may be that current and future advances in behavioral and molecular methods could position neurotoxicologists to make significant inroads towards identifying, describing, and possibly predicting these types of neurotoxicity in a manner that is more useful for risk assessors. As methods for identifying and predicting silent neurotoxicity caused by environmental agents are advanced, it is important to keep in mind the lessons learned during the development of related theories (e.g., the "2-hit", neurodevelopmental hypothesis for schizophrenia; (Weinberger, 1996)). It is notable that, while the application of the "multiple hit" theory to nervous system effects, for schizophrenia in particular, has now gained wider acceptance, it was initially met with criticism and sparked controversy. The controversy was primarily a result of inconsistencies in individual study findings and an initial inability to plausibly link neurodevelopmental abnormalities to clinical symptoms after a delay of decades; more recent studies have strengthened the coherence of the findings (Weinberger, 1996).

The panelists felt it important to convey how little we still understand about silent neurotoxicity. There are some data which begin to describe the potential use of markers of inflammation or immune response (e.g., cytokines such as interleukin-6), glial activation, or markers of oxidative stress, to detect early changes that might be associated with silent neurotoxicity. For example, recent publications from two of the panelists suggest that changes in microglial- and/or astrocyte-specific proteins can be observed in those exposure groups and sexes demonstrating changes in behavior due to a combination of chemical and nonchemical challenges (Allen et al., 2014; Bolton et al., 2014). Interestingly, these alterations were dependent on both the brain region(s) and behavior(s) being examined. However, the data, and our ability to interpret what these data indicate, are far from complete. In particular, the temporal progression of these preliminary associations (e.g., whether early changes in neuroinflammatory markers might predict future vulnerability to a subsequent neurotoxicant challenge) has not yet been examined. Given the biological complexity of these types of responses, it is unlikely that risk assessors will have data sufficient to provide a clear mechanistic understanding of any observed changes. This may be important to keep in mind when drawing conclusions regarding, for example, the consistency or dose-dependency of the data. For example, evidence for behavioral changes in only one sex, or at only one exposure level, or at only one time point, with data indicating these behaviors are not changed when these conditions are varied, should not necessarily be viewed as inconsistent or in conflict. In such cases, it is important to carefully consider both the strength of the evidence (e.g., replicated findings would be interpreted with greater confidence) and whether methodological differences may exist to explain discrepancies across studies. While an understanding of the mechanism(s) for toxicity can aid interpretation, lack of a mechanistic explanation for what might be a very complicated biological association is generally not reason enough to discount these types of findings. Unfortunately, we do not currently have any molecular, morphological, or behavioral features that risk assessors could use as effective indicators or biomarkers for when particular types of silent damage have occurred. As previously noted by Giordano and Costa (2012), knowledge regarding potential mechanism(s) of

developmental neurotoxicity would help aid the understanding of silent neurotoxicity, although it is important to recognize that these authors also noted that this knowledge is not a requirement for risk assessment.

It is clear that traditional test guidelines are generally incapable of revealing or detecting these types of effects. The panelists argued that there is a need for toxicologists to better consider the intricacies of human neurotoxicity, both in the context of exposure and co-occurring risk factors as well as the complexity of human behavior. Similarly, risk assessors were encouraged to pay particular attention to the study-specific parameters that might influence the detection sensitivity (or lack thereof) of a particular experiment. These parameters include the timing of exposure and latency before behavioral testing, the types and timing of unmasking challenges, whether the specific characteristics of the experimental animals encompass potentially-vulnerable populations, and how well the behavioral assays serve to investigate a diverse array of human behaviors. Generally, to reveal silent neurotoxicity, experiments should be designed in such a way that they are capable of challenging or pushing the threshold for a particular response of the system, such that modest effects on that system (e.g., capacity-changing effects due to exposure) have the potential to be revealed. As these types of studies become more commonplace, continued discussion and coordination between neurotoxicology researchers and risk assessors (e.g., through training opportunities or collaborations on test design and interpretation) may help to reduce uncertainties related to evaluating the potential for silent neurotoxicity in human health risk assessments of environmental toxicants. However, given the complexity of these experiments, both in conduct and interpretation, there is a future need to better clarify the signals or situations that might suggest an elevated concern for potential silent neurotoxicity.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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